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Evidence of NK1 and NK2 Tachykinin Receptors and their Involvement in Histamine Release in a Murine Mast Cell Line

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Abstract—Binding of [^{3}H]substance P (SP) and histamine release were examined using a cloned mouse mast cell line. SP binding was saturable and specific. In the presence of 30 mM Na_2SO_4/50 mM Tris buffer, SP interacted with two types of binding sites with K_d values of 0.3 and 40 nM. High-affinity SP binding was blocked by the inclusion of 0.5 uM of the NK1 receptor selective ligand septide in the binding mixture. Neurokinin A (NKA) evoked concentration-dependent histamine release. At concentrations in the nanomolar range, the NK1 preferring agonists SP, SP methylester and physalaemin evoked ≤5% net release of histamine, which was substantially less than the maximum effect of NKA (+37%) in the micromolar range. Pretreatment of the cells with the NK2 antagonist peptide A reduced NKA-induced histamine release. [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]-substance P, a putative SP antagonist, also elicited histamine release in the micromolar range, apparently acting as an agonist at the NK2 site. Compound 48/80, N-terminal SP fragments, neurokinin B and the two selective NK2 receptor antagonists cyclo(Gln-Trp-Phe-(R)-[Ala-2]Leu-Met) (peptide A) and cyclo(Gln-Trp-Phe-Gly-Leu-Met) (peptide B) were ineffective. Although the results suggest the coexistence of functional NK1 and NK2 receptors, it appears that in this mast cell line neurokinin-induced histamine release is primarily mediated by the NK2 receptor, characterized biochemically as a low affinity binding site with a K_d value of 40 nM for SP.

Introduction

The undecapeptide substance P (SP), considered to be a neurotransmitter involved in sensory processes, is now thought to cause a wide range of peripheral effects, e.g., vasodilation, plasma extravasation and immunomodulation (1). SP belongs to the peptide family known as tachykinins, characterized by a common C-terminal sequence, Phe-X-Gly-Leu-Met-NH_2 (Table 1). In addition to SP, two other mammalian tachykinins, named neurokinins, are known, the decapeptides neurokinin A (NKA) and neurokinin B (NKB) (2). The biological effects of SP, NKA and NKB are mediated through their respective tachykinin receptors, NK1, NK2 and NK3 (2-4). Among the

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Table 1 Amino acid sequences of the mammalian tachykinins (neurokinins).

Substance P (SP)	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
Neurokinin A (NKA)	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
Neurokinin B (NKB)	Asp-Met-His-Asp-Phe-Val-Gly-Leu-Met-NH_2

three neurokinins, only SP is known to induce histamine release from some types of mast cells, such as rat peritoneal (5, 6) and human skin mast cells (7, 8). SP action on mast cells is not fully understood. Whereas most of the biological effects of neurokinins depend on the interaction of the common C-terminal sequence (4), the histamine releasing activity of SP, in contrast, requires the basic N-terminal domain of the SP molecule (9, 10), i.e., Arg-Pro-Lys-Pro. However, interactions of the C-terminal portion are needed for maximum histamine release (11). Because receptors on mast cells recognizing the N-terminal domain of the SP molecule have not yet been identified (12), mediation of SP action via such receptors is presently being questioned. Other mechanisms for SP action on mast cells have been suggested. For example, a receptor-independent pathway involving GTP-binding regulatory proteins (G proteins) has been suggested as a common mechanism for the histamine releasing action of SP and the poly-basic molecules mastoparan and compound 48/80 (12-14). Mastoparan, a peptide toxin from wasp venom, and compound 48/80 are potent histamine releasers in rat peritoneal mast cells (11, 13) but not in guinea pig mast cells (15), human pulmonary mast cells (16) or human basophilic leukocytes (17, 18). SP displays similar variation in evoking histamine release from mast cells of different tissues and species (5-11, 13, 19). These variations in response remain inexplicable.

In this report, the action of neurokinins on a cloned mouse mast cell line, MC/9, derived from fetal liver cells from a (B6 X A/J)F_1 mouse (20), is described. The aim of this study was to examine biochemically SP binding, using a homogeneous cell population, and correlate the binding pattern with selective neurokinin-induced histamine release to 1) identify the tachykinin receptors present on mast cells and 2) assess their role in neurokinin-induced histamine release. Biochemical studies of SP binding to mast cells are lacking. Most studies of SP-induced histamine release have been performed on heterogeneous but enriched populations of rat peritoneal mast cells or blood basophils, not well suited for biochemical assays. Autoradiographic studies of SP binding have failed to show specific binding of SP to rat peritoneal mast cells (21).

In pilot experiments, NKA was found to release histamine from MC/9 cells, while SP, N-terminal SP fragments, compound 48/80 and the mast cell degranulating peptide (MCD), another potent histamine releasing compound isolated from wasp venom, caused little or no histamine release. Moreover, specific binding of SP to MC/9 cells was observed. These experiments were expanded and are described in detail here. A part of this investigation has previously been presented in an abstract (22).

Materials and Methods

Materials

Radioligand used was [2-prolyl-3,4-^{3}H]substance P ([^{3}H]SP; 36.3 and 42.6 Ci/mmol, New England Nuclear, MA). Unlabeled peptides were purchased from Peninsula Laboratories, CA, or Cambridge Research Biochemicals, NY, and mast cell degranulating peptide (MCD) was from Bachem, CA. Bovine albumin, Fraction V, bacitracin, bestatin, compound 48/80 and histamine diphosphate salt were purchased from Sigma Chemical Company, MO. Fluoraldehyde o-Phthalaldehyde reagent solution was purchased from Pierce Chemical Company, IL. The MC/9 mast cell line was purchased from American Type Culture Collection (ATCC, CRL 8306), MD.

Methods

The MC/9 cells were incubated as suspension cultures in Dulbecco's modified Eagle's medium

(DME) containing L-arginine HCl (116mg/l), L-asparagine (36mg/l), folic acid (6mg/l), non-essential amino acids (0.1mM), sodium pyruvate (1.0mM) and fetal bovine serum (10%), supplied by ATCC. Actively growing cultures, seeded at 10^5cells/ml in Corning 25cm^2 polystyrene tissue culture flasks and supplemented with fresh culture medium every 2-3 days, were allowed to multiply at 37°C in a 7% CO_2-air mixture for 5 to 6 days. The cultures were harvested at a cell density of 1.5 $\times$ 10^6cells/ml. Aliquots of the cultured cells were prepared for histamine release studies, while remaining cells were pelleted and stored at −70°C or in liquid nitrogen. Cell viability was assessed by the Trypan blue exclusion test. Cultured cells of viability ≥75% were used in the experiments.

Binding studies. A modification of the method described by Maruyama (23) was used. The frozen cell pellets were thawed and diluted with 50 volumes of ice-cold 50mM Tris-HCl buffer (pH 7.4), vortexed vigorously, then centrifuged at 40000g for 15mn at 4°C and washed one more time. The resulting pellet was resuspended in Tris buffer (600mg protein/ml), now containing bacitracin (final concentration 40ug/ml), and distributed in 0.25ml aliquots to a set of polypropylene tubes containing 40uM bestatin, 0.4mg bovine albumin and with or without 30mM Na_2SO_4 (total volume 1ml). For binding saturation analysis, increasing concentrations of [^{3}H]SP were added in the presence and absence of 1uM of unlabeled SP. In competitive inhibition studies, increasing concentrations (0.1nM-10uM) of unlabeled SP were added in the presence of a fixed concentration of [^{3}H]SP (0.2-2nM). After 20mn incubation on ice, the labeled membranes were collected by filtration over Whatman GF/B filters under vacuum, followed by three rapid washes with ice-cold Tris buffer using the M-24R Cell-Harvester (Brandel Instruments). Both the tubes and the filters were soaked with 0.1% polyethylenimine for 24h before use. The binding of [^{3}H]SP to crude MC/9 membranes was determined from the difference between parallel experiments with membranes or the vehicle for each combined concentration of labeled and unlabeled SP to rule out contributions of [^{3}H]SP binding to non-biological materials. Binding data were analyzed using the LIGAND computer program (24) to obtain estimates for the apparent equilibrium dissociation constant, K_d, and maximum binding capacity, B_{max}. Models involving one or two independent classes of binding sites were evaluated for each experiment. The most appropriate model was shown on the basis of the 'extra sum-of-squares' F-test. The protein concentration was determined by Peterson's modification of the micro-Lowry method (Sigma Protein Assay Kit) using bovine serum albumin as a standard.

Histamine release studies. Cultured cells were pelleted, washed twice and resuspended in Tyrode solution of the following composition (mM): NaCl 137, KCl 2.7, NaH_2PO_4 0.4, $MgCl_2$ 1, glucose 5.6, HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid) 10, and with or without $CaCl_2$ 1.8mM (pH 7.4, 20°C). Aliquots (0.9ml) of cell suspensions (10^6cells/ml), subsequent to temperature equilibration (5min, 37°C), were incubated with the indicated concentrations of a possible releasing agent or the vehicle (spontaneous release) in a volume of 0.1ml. The reaction was stopped after 15min incubation by chilling the tubes in an ice bath. The cells were separated from the medium by centrifugation for 10min at 300g, 4°C. Released histamine was measured in the supernatants. Residual histamine in the cells was measured after lysing the cells by addition of distilled water to the pellets followed by protein precipitation with 20% tricholoroacetic acid and centrifugation at 1000g. Histamine was assayed fluorimetrically by the method of Shore et al. (25) using an excitation wavelength of 348nm and emission wavelength of 439nm. Histamine standard ranging from 25ng/ml to 250ug/ml were used for calibration of the assay. Histamine released in the supernatant was expressed as a percentage of total histamine present in the cells plus supernatant. Spontaneous release in the absence of a releasing agent was subtracted from the stimulated release to estimate net induced release. The releasing agents were examined for possible interference with histamine determination, and corrections were made when required

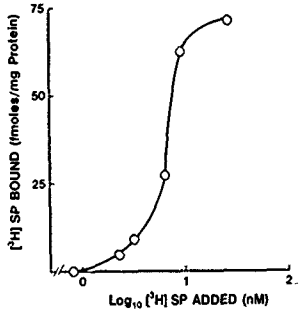

Fig. 1 Concentration dependence of the specific binding of [^{3}H]SP to mast cell membranes in 50mM Tris buffer, 4°C. All values are the mean of quadruple samples. The difference between samples were less than 18%.

Results

Binding of [^{3}H]substance P to mast cell membranes. The binding of [^{3}H]SP to mast cell membranes was rapid. Time course studies of 0.2nM [^{3}H]SP binding revealed that maximum specific binding was reached after 20min incubation at 4°C (data not shown). After 1h incubation, 87% of maximum binding remained bound, while after 3h incubation, 72% remained bound. Hence, 20min incubation was routinely used. Figure 1 demonstrates that specific binding of [^{3}H]SP is saturable. Artifactual non-receptor interactions were presumably absent since background counts were subtracted from each assay point (see Methods). The specific binding, obtained by deducting non-specific binding in the presence of 1uM unlabeled SP from that of total binding without SP, was only 10 to 15% of total binding. For example, the addition of 18nM [^{3}H]SP (797300cpm) resulted in total [^{3}H]SP binding of 11154cpm and specific [^{3}H]SP binding of 1267cpm (2mL total volume of binding mixture) after binding to nonbiological substances had been accounted for.

The shape of the saturation curve is indicative of cooperative binding. For positive cooperativity, the ascending portion of the curve is almost parallel with the y-axis (26), here indicated by [^{3}H]SP bound. Facilitated or cooperative binding results in concave downward Scatchard plots (not shown), in which K_d varies with amount bound. Therefore, the parameters for the binding of [^{3}H]SP were not calculated based on these binding data.

Competitive inhibition studies of [^{3}H]SP binding by increasing concentrations of unlabeled SP were performed in high ionic strength solutions by the inclusion of Na_2SO_4 in the binding mixtures, which according to Bahouth and Masacchio (27) acts mainly by reducing nonspecific SP binding. The lowering of nonspecific SP binding was confirmed. The addition of 30mM Na_2SO_4 to the binding mixture produced nonspecific binding as low as 35% of total binding and was routinely used in the competition experiments. The binding parameters were estimated from the displacement of 2nM [^{3}H]SP binding (88000cpm) by increasing concentrations of unlabeled SP. The radioligand was used at the lowest practical concentration for detection of binding to sites of disparate characteristics. This concentration provided about 1200cpm of total binding and about 800cpm of specific binding after corrections for [^{3}H]SP binding to non-biological material. The displacement curve with the 95% confidence limit curves (Fig. 2) represents a conglomerate plot of binding data from four separate experiments. A two-site model was found to be a best fit for [^{3}H]SP binding to mast cell membranes. The estimated binding parameters are shown in Table 2.

Blocking studies with the highly selective NK1 receptor ligand [pGlu^6,Pro^9]substance P (septide) (28) were performed to further examine the heterogeneity of SP binding sites. Fig. 3 shows the effect of 0.5uM septide on the displacement curve in the

Table 2 Parameters for substance P binding to mast cell (MC/9) membranes in the presence of 30mM Na_2SO_4

	Apparent K_d (nM)	*B_{max} fmoles/mg protein*
High-affinity site	0.3	67
Low-affinity site	40.0	2700

Binding parameters were derived from the combined analysis of four displacement experiments using the LIGAND program (24). Data are shown in Figure 2.

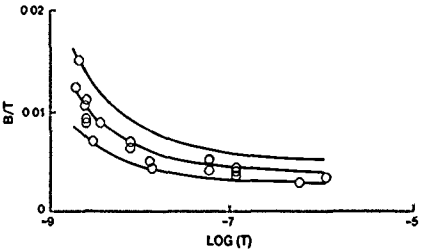

Fig. 2 Displacements of the binding of 2nM [^{3}H]SP by unlabeled SP from mast cell membranes in the presence of 30mM Na_2SO_4. The displacement curve with 95% confidence limit curves is a conglomerate plot based on four competition experiments, indicating binding to two sites (Table 2). B/T = bound divided by total radioactivity; log (T) = logarithm of the total ligand concentration.

presence of 30mM Na^2SO_4. The absence of high-affinity displacement of [^{3}H]SP binding, shown by an initial plateau in the plot, is indicative of septide blocking of NK1 sites.

Release of histamine from mast cells. The amount of histamine in the MC/9 cells varied with number of days in culture. A maximum amount up to 0.608ug/10^6 cells was measured, which was more than 10-fold that of mast cells derived from murine bone marrow cultures (29), 0.053ug/10^6 cells, but much lower than that of either human pulmonary, 4.23ug/10^6 cells, or rat peritoneal mast cells, 30ug/10^6 cells, (16).

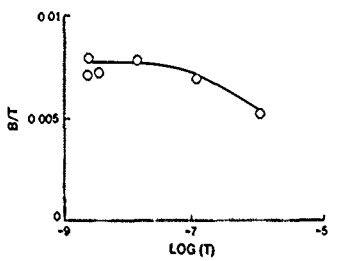

Fig. 3 Representative curve of the displacements of the binding of 2nM [^{3}H]SP by unlabeled SP from mast cell membranes in the presence of both 30mM Na_2SO_4 and 0·5uM septide. Analysis of binding data was based on duplicate measurements, and the experiment was repeated once with similar results.

Table 3 Histamine release from mast cells (MC/9) induced by tachykinin receptor ligands

Ligand	*Conc ($M \times 10^{-6}$)*	*(n)*	*Histamine release (% of total)*
Substance P (SP)[a]	0·010	(3)	5 ± 1
"	0·100	(1)	4
"	1·0	(1)	3
"	10·0	(1)	3
"	100·0	(2)	4 ± 0·7
SP methylester	0·010	(3)	3 ± 1
"	0·100	(2)	4 ± 0·7
Physalaemin[a]	0·010	(5)	4 ± 0·7
Neurokinin A (NKA)	0·010	(1)	0
"	0·1	(2)	2 ± 0·7
"	1	(10)	4 ± 0·6
"	10	(5)	7 ± 1
"	20	(2)	10 ± 0·7
"	50	(3)	13 ± 0·7
"	100	(5)	37 ± 5
[D-Arg^1,D-Phe^5,	0·01	(1)	0
"	0·10	(1)	0
"	1	(1)	3
D-Trp^{7,9},Leu^{11}]-	10	(2)	4 ± 2
substance P	50	(2)	13 ± 0·7
(peptide X)[b]	100	(2)	41 ± 0

The values, expressed as Mean ± SEM ($n \geq 3$) and Mean ± SD ($n = 2$), represent induced release, which is ligand stimulated release minus spontaneous release.
[a]Detectable in the absence of extracellular $CaCl_2$. [b]Potent bombesin antagonist (30) and putative SP antagonist (31)

Histamine was released from suspensions of MC/9 cells by micromolar concentrations of the NK2 preferring agonist, NKA, and only to a minor degree by nanomolar concentrations of the NK1 selective agonists, SP, SP methylester and the amphibian tachykinin, physalaemin; (Table 3). Increasing concentrations of the NK1 preferring agonists up to 100uM did not produce an increase in histamine release. Septide appeared to have no histamine releasing capability. To detect induced release for SP and physalaemin, it was necessary to omit extra-cellular $CaCl_2$ from the medium. This is a condition the MC/9 cells share with rat peritoneal mast cells (10). Omitting $CaCl_2$ extra-cellularly yielded an overall slightly lower value for the spontaneous release, 16 ± 1% (n = 16), as

compared to 18 ± 1% (n = 32) in the presence of 1.8 mM $CaCl_2$, but had little or no effect on induced histamine release except when SP or physalaemin were the releasers. Extending the incubation period with the releasing agents from 15 min up to 60 min did not change the values of the spontaneous release. Induced histamine release reached a maximum at 7.5 min incubation and remained constant up to 60 min, the longest period examined. [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]] substance P (peptide X), a potent bombesin antagonist (30) and putative SP antagonist (31) induced the release of histamine in a similar concentration range as did NKA, while two selective antagonists for the NK2 receptor (32), cyclo(Gln-Trp-Phe-(R)Gly-[ANC-2]Leu-Met) (peptide A) and cyclo(Gln-Trp-Phe-Gly-Leu-Met) (peptide B), were without effect up to 1000 uM. Compound 48/80, examined from 0.001 to 1000 ug/ml, a range known to cause release in rat peritoneal mast cells (10, 11, 13), MCD at 1 uM, the N-terminal SP fragments, SP(1-4) (0.01-100 uM) and SP(1-6), SP(1-7) or SP(1-9) at 100 uM, as well as NKB, a preferential NK3 receptor agonist, in the range 0.01-35 uM, evoked little or no histamine release (data not shown). The two NK2 selective antagonists, peptide A and B (see above), were examined for their ability to inhibit NKA-induced histamine release. Peptide A appeared to be most effective. Preincubation of the cells with either 10 or 100 uM of peptide A for 10 min at 37°C prior to the exposure to equimolar concentrations of NKA reduced the histamine release by 25 and 57%, respectively. Preincubation with the SP antagonist (33), [D-Arg[1],D-Trp[7,9],Leu[11]]substance P (spantide), on the other hand, had no reducing effect.

The MC/9 cells, like rat peritoneal mast cells (34), released histamine with concanavalin A (con A), an IgE-type secretagogue. Incubation for 15 mun with 2.5 and 5 ug/ml of con A without the potentiator phosphatidylserine evoked 11% and 9% induced release, respectively.

Discussion

The results demonstrate th the MC/9 mast cell line possesses specific binding sites for SP. Moreover analysis of the binding data has revealed that SP binds to two types of sites of vastly different binding properties. As SP interacts with all three tachykinin receptors, but preferentially with the NK1 type, the high-affinity binding is compatible with that of SP binding to NK1 receptors. While blocking of high-affinity SP binding using the selective NK1 ligand septide (Fig. 3) confirms the presence of NK1 receptors, the functional studies support the presence of NK2 receptors.

We have demonstrated here that none of the well-known histamine releasers, compound 48/80, MCD and N-terminal SP fragments evoked histamine release from the MC/9 cells. On the other hand, the results have revealed the novel observation that NKA acts as a histamine secretagogue in this cell type. Moreover, the reduction in NKA-induced histamine release observed after pretreatment with a NK2 antagonist but not with a NK1 antagonist (spantide) suggests the involvement of NK2 receptors in this process and thereby the existence of NK2 receptors on this cell line. The weak activity of the selective NK1 agonists suggests the presence of a minor population of functional NK1 sites. However, the involvement of NK1 receptors in NKA-induced histamine release was dismissed on the following grounds: (1) NKA is shown to interact preferentially with NK2 receptors; (2) pretreatment of the cells with the SP (NK1) antagonist spantide led to no loss in NKA-induced histamine release; while (3) pretreatment with a NK2 antagonist did. A lack of effect by the NK3-preferring ligand NKB precluded the possible involvement of NK3 receptors in NKA-induced histamine release.

Recently, pharmacological and biochemical evidence has suggested that there are multiple subtypes of NK2 receptors (35). A series of linear and cyclic NK2 antagonists were found to have varying potency in different NK2 containing tissues. The finding that the selective NK2 receptor antagonist, peptide A, inhibited only partially NKA-induced histamine release might indicate the presence of a heterogenous pool of NK2 sites on MC/9 cells.

The present studies have provided evidence for the existence of NK1 and NK2 receptors on the MC/9 cell line. The coexistence of physiologically relevant NK1 and NK2 receptors has recently been described for different functions in other types of

tissues. For example, both SP and NKA were found to stimulate the release of 5-hydroxytryptamine via NK1 and NK2 receptors in the rat cerebral cortex (33). Both receptor types have also been implicated in SP-induced superoxide anion production in guinea-pig alveolar macrophages (36).

It has been argued that, because some SP antagonists, such as [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]substance P, elicit histamine release (37), the induction of histamine release by SP is not tachykinin receptor-mediated. Because several antagonists of SP with two or more D-Trp residues of limited selectivity for all three tachykinin receptors have considerable agonist properties for these sites (38, 39), it is possible that we deal with a class of compounds with mixed agonist-antagonist properties. Peptide X (Table 3) is apparently such a compound. This peptide described initially as a SP antagonist that blocks the hyoscine-resistant opiate withdrawal contracture in guinea pig ileum (32), was later found to be a potent antagonist of the amphibian tetradecapeptide bombesin and structurally related mammalian peptides, including gastrin-releasing peptide (GRP), (31). Peptide X has also been found to inhibit the action of the neurohypophyseal hormone vasopressin (40). Since vasopressin and the peptides of the bombesin family interact with distinct and specific high-affinity receptors, it appears that peptide X interacts as a blocking agent with several independent receptors. However, present studies indicate that peptide X, which has minimal structural homology with NKA (only Leu^{10}), elicits a biological response. Thus, peptide X functions apparently as a mixed agonist-antagonist, expressing agonist characteristics at the NK2 receptor and antagonist characteristics at various independent receptors, including the NK1 tachykinin receptor. In conclusion, this study has demonstrated the presence of both NK1 and NK2 receptors on a murine liver derives mast cell line and is the first to show that histamine is released by NKA via NK2 tachykinin receptor activation. Whereas septide appears to have no histamine releasing activity in this cell type, other NK1 preferring agonists, e.g., SP methylester, SP and physalaemin, exert a minor stimulating effect on such release, suggesting the possible involvement of NK1 receptors. We have shown here that in a mast cell type, unresponsive to known basic secretagaogues such as compound 48/80 and N-terminal SP fragments, a NK2 receptor-dependent pathway might exist for the release of histamine.

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